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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Appl. No : 10/815,727 Confirmation No. : 9476  
Applicant : John D. Brennan et al.  
Filed : April 2, 2004  
Title : METHOD OF IMMOBILIZING MEMBRANE-ASSOCIATED  
MOLECULES  
TC./A.U. : 1841  
Examiner : Unsu Jung  
Docket No. : 3244-127 (Formerly 571-933)  
Customer No: 001059

Honorable Commissioner for Patents  
P. O. Box 1450  
Alexandria, Virginia 22313-1450

Dear Sir:

**DECLARATION UNDER 37 CFR §1.132**

I, Michael A. Brook, a citizen of Canada, and resident of Ancaster, Ontario, Canada, declare that the following facts are within my knowledge and are true.

1. I reside at 165 Charterhouse Crescent, Ancaster, Ontario, Canada L9G 4M4.
2. I currently am a Professor in the Department of Chemistry, McMaster University, 1280 Main St. W., Hamilton, Ontario, Canada, L8S 4M1.
3. I have been working in the area of organic, polymer and materials synthesis utilizing silicon chemistry since 1980. My curriculum vitae is attached to this Declaration as Exhibit A.

4. I am an Inventor, along with Zheng Zhang, Yang Chen, Jorge Cruz-Aguado, Richard J. Hodgson, Dina Tleugabulova and John D. Brennan, of the subject matter as claimed in U.S. Patent Application No. 10/814,123 filed April 4, 2004 (hereafter "the Application").

5. I have read and understood the disclosure and claims of the Application.

6. I have read and understood the Office Action that issued on the Application on May 17, 2008. The Examiner is of the view that claims 1-5, 8-10, 38, 40-45 and 47-48 are obvious over Nakanishi688 (US 5,009,688) in view of Gill (J. Am. Chem. Soc., (1998), 120, 8587-8598), claims 1-5, 8-10, 40-45, 47-52, 54-55 and 56 are obvious over Nakanishi875 (US 5,624,875) in view of Gill, claim 38 is obvious over Nakanishi875 in view of Gill and as evidenced by Barkin (US 3,374,103) and claims 53 and 57-61 are obvious over Nakanishi875 in view of Gill.

7. I have read and understood the claims that are attached to this Declaration as Exhibit B that I understand the Applicants are filing in response to the Office Action dated May 17, 2008. My comments below are based on the amended claims in Exhibit B (hereinafter "the amended claims").

8. The Applicants have developed a biomolecule compatible method of preparing bimodal siliceous materials having a meso/macroporous structure that is suitable for chromatographic applications by combining polyol-modified silane precursors with one or more water soluble polymers under conditions where a phase separation occurs before gelation, wherein said conditions comprise combining polyol-modified silane precursors with one or more water soluble polymers at a pH in the range of about 4 to about 11.5.

9. Nakanishi688 describes methods of preparing siliceous materials with controlled pore size by combining alkoxysilanes, or oligomers thereof, and a

water soluble polymer, under conditions where phase separation occurs before gelation. Nakanishi688 does not teach that the resulting materials are bimodal, i.e. that they have a meso/macroporous structure. The materials prepared using the method taught in Nakanishi688 are only described as "porous".

10. Nakanishi875 describes methods of preparing siliceous materials with a bimodal meso/macroporous pore structure by combining alkoxysilanes, or oligomers thereof, and a water soluble polymer, under conditions where phase separation occurs at least concurrently with gelation, followed by treatment of the resulting gel with a matrix dissolving agent. Nakanishi875 does not teach that bimodal (i.e. meso/macroporous) silica materials can be obtained by hydrolyzing and condensing an alkoxysilane in the presence of a water soluble polymer. The bimodal structure is obtained only after treatment of the gel with a matrix dissolving agent.

11. Gill describes methods of entrapping biomolecules in siliceous materials prepared from oligomeric polyol silicates such as polyglyceryl silicate (PGS). PGS was prepared by the partial hydrolysis and condensation of tetramethyl orthosilicate (TMOS) to poly(methyl silicate) (PMS), followed by transesterification with glycerol, in a one pot reaction catalyzed by hydrochloric acid or poly(antimony(III) ethylene glycoxide). Specifically, at page 8595-8596, Gill describes the preparation of methyl/ethyl ester and polyol ester precursors as follows:

Poly(methyl silicate) (PMS) and poly(glyceryl silicate) (PGS): TEOS (0.48 mol) was mixed with ethanol (50 mL), and hydrochloric acid (10.4 mL of 0.25 M) was added over 30 min with vigorous stirring; then the mixture was heated to 70 °C for 15 h. Rotary evaporation at 35 °C provided PMS of composition  $\text{SiO}_{1.1-1.2}(\text{OMe})_{1.6-1.8}$  as a clear, viscous liquid. PGS was obtained by adding glycerol (0.38 mol) to the reaction mixture over 1 h, heating to 50 °C, and stirring for a further 40 h. [...] FAB-MS indicated that the product consisted mostly of glyceryl-bridged linear oligomeric polysilicates of DP 5-9.

Various glyceryl silicates ("SiGlc<sub>2-4</sub>") and poly(glyceryl silicates) ("SiO<sub>0.5-1.5</sub>-Glc<sub>0.5-2</sub>") were prepared by this method.

Gill utilizes Bronsted (HCl) or Lewis (poly(antimony(III) ethylene glycoxide)) acid catalysts and water to prepare PGS. Such conditions are ideal for alkoxysilane hydrolysis and, ultimately, condensation to prepare siloxane oligomers and polymers. Gill notes that DP 5-09 oligomers are formed. Thus, Gill prepares mixed alkoxy / siloxy species that he calls PGS. It is not possible to prepare pure alkoxysilanes in a medium containing water, such as hydrochloric acid, particularly when acidic catalysts are present (see C. J. Brinker and G. W. Scherer, *Sol-Gel Science - The Physics and Chemistry of Sol-Gel Processing*, New York, Academic Press, 1990 - p. 116 "Tetraalkoxysilanes, organotrialkoxysilanes, and diorganodialkoxysilanes hydrolyze upon exposure to water vapor"; "Hydrolysis is most rapid and complete when catalysts are employed."; "Many authors report that mineral acids are more effective catalysts...").

12. Diglyceryl silane (DGS) is an example of a polyol-modified silane precursor.

13. We have performed direct side-by-side comparison hydrolysis and condensation reactions of DGS, PGS and TEOS in the presence of polyethylene oxide (PEO, 10K MW) with or without added glycerol. Reactions were performed at pH 5.5 and at pH 11 which represent the ends of the pH ranges that are claimed in the application. The reaction conditions, with the exception of pH, are commensurate in scope with those taught in Nakanishi688 or Nakanishi875 in view of Gill. Experimental details and scanning electron microscopy (SEM) images of the resulting materials are presented as Exhibit C.

14. The results provided in Exhibit C show that the DGS samples 1, 5, 6 exhibit macroporosity and (not shown) mesoporosity. The morphology of the structures varies, but is in all cases open. Sample 2 is not macroporous. Under

these conditions, the gelation occurred prior to phase separation. In order to slow down gelation, one equivalent of glycerol was added while other conditions were kept constant. The retarded hydrolysis rate led to phase separation *prior* to gelation and a macroporous structure was achieved (sample 6). To more broadly show the effect of changing the rate, 1 equivalent of glycerol was added to all of DGS, TEOS and PGS reactions (samples 5, 6, 7, 8, 11 and 12). As can be clearly seen, under these conditions only DGS at either pH 5.5 or pH 11 led to macroporous structures, while TEOS and PGS did not. This demonstrates the significance of the pH ranges claimed in the application.

The SEM pictures of TEOS derived silica show that macroporous structures are not formed: with glycerol present, a 2 phase system results that does not cure within 1 day.

PGS does not lead to macroporous silica, irrespective of the presence of glycerol.

15. The experimental results show that DGS, used in the methods claimed by the present Applicants is fundamentally different from the material(s) prepared in Gill, Nakanishi688 and Nakanishi875. Specifically, in the presence of PEO (10K MW), DGS was the only precursor that provided macroporous material. Accordingly, DGS is not equivalent to PGS or TEOS. Further, in the presence of glycerol and PEO (10K MW) DGS was again, the only precursor that provided macroporous material. Accordingly DGS is not equivalent to PGS plus glycerol or TEOS plus glycerol.

16. In summary, I believe that Applicants are entitled to claim a method of preparing bimodal siliceous material by combining polyol-modified silanes with one or more water soluble polymers under conditions where a phase separation occurs before gelation as specified in the amended claims. I am of the opinion that the amended claims are not obvious in view of Gill in combination with

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### Introduction

- Membrane proteins comprise a large fraction of new drug targets
- Isolation and immobilization of these targets is vital for screening for new potential drugs
- Immobilization of model membrane proteins has been tremendously difficult due to the fragile nature of their structure
- Gramicidin provides a simple and ideal model system for the development of a sol-gel immobilization technique

### Properties of Gramicidin

- Monomeric spanning hydrophobic polypeptide
- Forms a cation selective ion-conducting channel
- Typical forms are parallel dimer with minor lipid flexibility, and a pore diameter that is known for passage of small ions
- Gramicidin has large influence on membrane lipid types and organization
- Solubility in organic solvents is low, but it is soluble in lipid bilayers
- Length is well suited for grafting onto a surface

### Tryptophan Fluorescence

- Gramicidin contains four tryptophan residues that are very sensitive to local environment
- Use shift in fluorescence emission as a probe for movement to a more non-polar environment
- Due to highly folded and scattering liposome samples accurate spectra are difficult to obtain. Preliminary results show red-shift in the emission spectrum

### Acknowledgments

MDS SOLEK  
J.D. Brennan thanks the Canada Research Chair in Biophysical Chemistry  
Sources: 1) Chemistry of Life, 2) E.C. Fowler, J. Chem. Soc. Faraday Trans. 2, 1976, 72, 1155  
3) J. J. Gray, J. Chem. Soc. Faraday Trans. 2, 1976, 72, 1155

### The Sol-Gel Process

- Hydrolysis  
 $\text{Si}(\text{OR})_4 + \text{H}_2\text{O} + \text{H}^+ \rightarrow \text{Si}(\text{OR})_3(\text{OH}) + \text{ROH}$
- Condensation  
 $2\text{Si}(\text{OR})_3(\text{OH}) \rightarrow (\text{OR})_3\text{Si}-\text{O}-\text{Si}(\text{OR})_3 + \text{H}_2\text{O}$
- Polycondensation  
 $n\text{Si}(\text{OR})_3(\text{OH}) \rightarrow (\text{Si}(\text{OR})_2\text{O})_n + n\text{H}_2\text{O}$
- Entrapment  
 $(\text{Si}(\text{OR})_2\text{O})_n + \text{Buffer} + \text{Liposomes} \rightarrow (\text{Si}(\text{OR})_2\text{O})_n + \text{Buffer} + \text{Liposomes}$
- Gelation  
Aging of monoliths or thin films followed by shrinkage

### Advantage of the Sol-Gel process vs. Surface Immobilization

Surface immobilization technique does not maintain natural liposomal environment

Sol-gel method allows entrapment of the entire vesicle with its lipid membrane and protein in its native form

### Monitoring Ion Mobility by Fluorescence

- Surfactin O's fluorescence intensity is sensitive to membrane potential
- Ion gradients must create net negative charge on the interior of the lipid membrane for a fluorescence signal to occur
- Gramicidin allows for passage of  $\text{K}^+$ , but not  $\text{Cl}^-$
- Liposomes must be created with high intrinsic KCl concentration
- Efflux of  $\text{K}^+$  causes membrane potential to increase, signaled by a increase in fluorescence intensity
- Same response can be measured for liposomes entrapped in sol-gel thin films

### Conclusions

- Intrinsic membrane protein Gramicidin has been successfully entrapped in sol-gel derived materials, which is demonstrated by Surfactin O membrane potential assay
- Physical and structural properties of Gramicidin in still unknown from preliminary chemist tryptophan emission spectra

### Future Work and Direction

- Investigation of peptide conformation by circular dichroism
- Study of known inhibitors of ion channel formation and/or function
- Monitor intrinsic tryptophan fluorescence lifetime of Gramicidin in sol-gel
- Electrochemical measurement method in other membrane receptors and enzymes